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PCT WORLD INTELLECTUAL PROPE
International Bu

INTERNATIONAL APPLICATION PUBLISHED UNDER

(51) International Patent Classification 6 :
A61K

A2

(11) In

WO 9607392A2

(43) International Publication Date: 14 March 1996 (14.03.96)

(21) International Application Number: PCT/US95/11477

(22) International Filing Date: 7 September 1995 (07.09.95)

(30) Priority Data:
08/302,132 7 September 1994 (07.09.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/302,132 (CIP)
Filed on 7 September 1994 (07.09.94)

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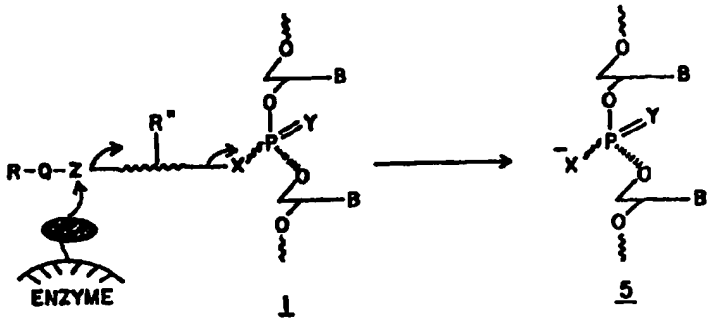
(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: OLIGONUCLEOTIDE PRODRUGS



Z = FUNCTIONAL GROUP

R = ALKYL, ARYL, AR-ALKYL, HETEROCYCLIC

R" = BRANCHING GROUP

X = O, S. NR (R = ALKYL, ARYL)

Y = O, S.

Q = HETEROATOM OR COVALENT BAND

(57) Abstract

Disclosed is an oligonucleotide prodrug comprising at least six covalently linked nucleotides, at least one of which is derivatized with a lipophilic chemical group reversibly and covalently attached to the nucleotide at a 5' phosphate, a 3' phosphate, or an internucleotidic phosphate linkage. The prodrug is reactive with a cellular or tissue enzyme which cleaves the lipophilic group from the derivatized nucleotide, thereby regenerating the parent oligonucleotide.

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OLIGONUCLEOTIDE PRODRUGS

BACKGROUND OF THE INVENTION

This invention relates to antisense therapy. More particularly, this invention relates to compositions and methods for enhancing the cellular uptake of antisense oligonucleotides.

New chemotherapeutic agents have been developed which are capable of modulating cellular and foreign gene expression. These agents, called antisense oligonucleotides, are single-stranded oligonucleotides which bind to a target nucleic acid molecules according to the Watson-Crick or Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of RNA by RNase H, or by destroying the target via reactive groups attached directly to the antisense oligonucleotide. Thus, they have become widely used research tools for inhibiting gene expression sequence specifically, and are under investigation for possible use as therapeutic agents (see, e.g., Lisciewicz et al. (*Proc. Natl. Acad. Sci. (USA)* (1993) 90:3860-3864); Bayever et al. (1992) *Antisense Res. Development* 2:109-110).

In order for antisense molecules to have therapeutic value, they must have the ability to enter a cell and contact target endogenous nucleic

acids. Furthermore, they must be able to withstand the rigors of the highly nucleolytic environment of the cell and/or body.

Recent studies have shown that oligonucleotides with certain modifications, such as artificial internucleotide linkages, not only render the oligonucleotides resistant to nucleolytic degradation (see, e.g., Agrawal et al. (1988) *Proc. Natl. Acad. Sci. (USA)* 85:7079-7083; Agrawal et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:7790-7794; Gao et al. (1990) *Antimicrob. Agents Chem.* 34:808; and Storey et al. (1991) *Nucleic Acids Res.* 19:4109), but also may increase cellular uptake of the oligonucleotide. For example, oligonucleotides with phosphorothioate or methylphosphonate internucleotide linkages have been found to bind to, and to be taken up by, cells more readily than phosphodiester-linked oligonucleotides (Zhao et al. (1993) *Antisense Res. Dev.* 3:53-56).

Oligonucleotide uptake is saturable, sequence-independent, and temperature and energy dependent. While there is some evidence to suggest that such uptake may occur through a 80,000 dalton membrane protein (Loke et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:3474; Yakubov et al. (1989) *Proc. Natl. Acad. Sci. (USA)* 86:6454), the gene for this protein has not yet been cloned or characterized. One study suggests internalization of the oligonucleotide is by a caveolar, protocytotic mechanism rather than by endocytosis (Zamecnick (1994) *Proc. Natl. Acad. Sci. (USA)* 91:3156).

Whether oligonucleotides are internalized via a receptor-mediated endocytotic pathway, a pinocytic mechanism, or a combination of both remains poorly understood.

To improve on their cellular uptake, oligonucleotides have also been modified in ways other than those described above. For example, an oligonucleotide with improved cellular uptake has been disclosed having at least one nucleotide residue covalently linked at its 2' position with various molecules including an amino acid, polypeptide, protein, sugar, sugar phosphate, neurotransmitter, hormone, cyclodextrin, starch, steroid, or vitamin (WO 93/23,570). Enhanced cellular uptake of biotinylated oligonucleotide in the presence of avidin has also been demonstrated (Pardridge et al. (1991) *FEBS Lett.* **288**:30-32). In addition, phosphodiester-linked oligodeoxynucleotides have been introduced into cells by the pore-forming agent streptolysin O (Barry et al. (1993) *Biotechniques* **15**:1016-1018), and a liposomal preparation including cationic lipid has been shown to enhance the cellular uptake of antisense molecules targeted to a portion of a human intercellular adhesion molecule (Bennett et al. (1992) *Mol. Pharmacol.* **41**:1023-1033). Phosphodiester-linked oligonucleotides bearing a 5'-cholesteryl modification show increased cellular uptake and biological effects (Krieg et al. (1993) *Proc. Natl. Acad. Sci. (USA)* **90**:1048). In addition, antibody-targeted liposomes have been used to enhance the cellular uptake of oligonucleotides targeted to HLA class I molecules

expressed by HIV-infected cells (Zelphati et al. (1993) *Antisense Res. Dev.* 3:323-338).

Specific non-oligonucleotidic, metabolically unstable molecules useful as medicaments have been prepared in the form of precursors or "prodrugs" which are capable of undergoing a chemical or enzyme-mediate transformation within the target organ or cell to release the therapeutic molecule (see, Bundgaard, in *Bio-reversible Carriers in Drug Design. Theory and Application* (Roche, ed.) Pergamon Press, NY (1987) pp. 13-94). For example, acyloxyalkyl ester-type groups have been appended to carboxylic groups of the β-lactam antibiotics such as pivampicillin, talampicillin, and bacampicillin to form prodrug derivatives of ampicillin (see, e.g., Daehne et al. (1970) *J. Med. Chem.* 13:607; Bodin et al. (1975) *Antimicrob. Agents Chemother.* 8:518; Clayton et al. (1976) *J. Med. Chem.* 19:1385). Phosphonate prodrugs of antiviral agents such as 9-[2-(phosphonomethyoxy)-ethyl]adenine (PMEA) (Starrett et al. (1994) *J. Med. Chem.* 37:1857-1864) and trisodium phosphonoformate (foscarnet sodium) (Iyer et al. (1989) *Tetrahedron Lett.* 30:7141-7144) have been prepared to increase oral availability. Phosphate groups have been appended to N-phosphomethyl dipeptides to form prodrugs of zinc protease neutral endopeptidase, an antihypertensive (De Lombaert et al. (1994) *J. Med. Chem.* 37:498-511). Anticancer prodrugs of butyric acid have been prepared (Nudelman et al. (1994) *J. Med. Chem.* 35:687-694). In addition, anti-herpes

prodrugs composed of diphosphate analogs of 5-iodo-2'-deoxy-uridine-5'-diphosphate have been reported (Jennings et al. (1992) *J. Chem. Soc. Perkin Trans.* I:2196-2202)

However, prodrugs of antisense oligonucleotides heretofore have not existed, and insufficient uptake of modified and unmodified oligonucleotides remains a problem both *in vitro* and *in vivo*. Thus, there remains a need for improved compositions and methods for enhancing the cellular uptake and metabolic stability of antisense oligonucleotides. Such enhancement would ultimately result in an increased efficacy of antisense oligonucleotides and a reduction in the dose administered that have to be used. Ideally, such compositions and methods will also be useful for increasing the general lipid solubility of oligonucleotides.

SUMMARY OF THE INVENTION

This invention provides improved compositions and methods for enhancing the cellular uptake and metabolic stability of antisense oligonucleotides, and for increasing the cellular and general *in vivo* lipid solubility of such oligonucleotides. Also provided are antisense oligonucleotides with enhanced cellular uptake, increased oral bioavailability, sustained or controlled release characteristics, reduced toxicity, and increased ability to cross physiological barriers.

It has been discovered that the *in vivo* half-life and uptake of antisense oligonucleotides into cells can be enhanced by the reversible derivatization of these oligonucleotides with a lipophilic chemical group. Covalent attachment of the lipophilic group to the oligonucleotides renders them less ionic and more susceptible to transport through of cell membranes than their underivatized parent. Once inside the cell or body, an endogenous enzyme cleaves the lipophilic group from the derivatized oligonucleotide, thereby regenerating the parent oligonucleotide. This discovery has been exploited to produce synthetic, reversibly derivatized antisense oligonucleotides or "oligonucleotide prodrugs" and methods of their use.

In one aspect of the invention, an oligonucleotide prodrug is provided. As used herein, the term "oligonucleotide prodrug" refers to a molecule including a plurality of nucleotides that are covalently linked together, 3' to 5', 5' to 3', 3' to 3', 5' to 2', 2' to 5, 2' to 3', or 3' to 2', and which has been masked or derivatized with a chemical group that causes the oligonucleotide to become more lipophilic, and hence to pass through lipid membranes with more ease than can the parent molecule. In addition, the oligonucleotide in its "prodrug" form may be less susceptible to degradation than its parent, but like its parent, may hybridize to other nucleic acids having a complementary nucleotide sequence. When in contact with certain enzymes in the cell, tissue, or body, the prodrug is cleaved

such that the parent oligonucleotide is regenerated.

The oligonucleotide prodrug includes at least six covalently linked nucleotides. At least one of these nucleotides is derivatized with a lipophilic chemical group reversibly and covalently attached to a 5' phosphate or a 3' phosphate of the nucleotide, or to an internucleotidic phosphate linkage.

As used herein, the term "nucleotide" refers to deoxyribonucleotides and analogs thereof, including analogs having a cyclic sugar and/or modified bases and riboxynucleotides and analogs thereof. In some embodiments, the oligonucleotide prodrug is "a hybrid oligonucleotide," i.e., it includes at least one ribonucleotide or analog thereof, and at least one deoxyribonucleotide or analog thereof. In one specific embodiment, the ribonucleotide analog is a 2-O-alkyl ribonucleotide such as a 2-O-methyl.

The lipophilic group attached to the nucleotide includes an ester or an amide, and the prodrug reacts with a cellular or tissue enzyme which cleaves the lipophilic group from the derivatized nucleotide. In preferred embodiments, the enzyme is an esterase if the lipophilic group comprises an ester, or is a phosphoramidase if the lipophilic group is an amide.

The lipophilic chemical group covalently attached to the nucleotide is an alkyl, aryl,

alkane, ar-alkyl, heterocyclic group, fatty acid, steroid ester, or steroid amide. In some preferred embodiments, if more than one nucleotide is derivatized, the chemical group attached thereto may be a mixture of these lipophilic groups. In other preferred embodiments, the lipophilic chemical group is attached to a sulfur, oxygen, or amine group on the 3' phosphate or the 5' phosphate of the nucleotide, or on an internucleotidic phosphate, or to a phosphorothioate, phosphorodithioate, phosphoramidate, or phosphate ester group on the nucleotide.

In other embodiments, the oligonucleotide prodrug is "chimeric". As used herein, "chimeric" refers to an oligonucleotide composed of more than one type of nucleotide. In one particular embodiment, the oligonucleotide prodrug consists of at least two different nucleotides such as a phosphodiester, carbamate, phosphorothioate, phosphorodithioate, acetamidate, phosphoramidate, phosphodiester, alkylphosphonate, carbonate, alkylphosphonothioate, phosphoramidite, or carboxymethyl ester, or any analog that is isosteric with the base sugar and internucleoside moiety of an unmodified oligonucleotide. In other embodiments, the oligonucleotide prodrug may be branched, i.e., may comprise two oligonucleotide sequences linked together via their 3' and/or 2' ends.

The invention also provides a pharmaceutical formulation including an oligonucleotide prodrug.

In some embodiments, this pharmaceutical formulation contains an oligonucleotide prodrug that is complementary to a region of a viral nucleic acid, and also contains another antiviral agent in addition to the prodrug. In one particular embodiment, the oligonucleotide prodrug in the pharmaceutical formulation is complementary to a first region of the viral nucleic acid, and the antiviral agent is an antisense oligonucleotide having a nucleotide sequence complementary to a second region of the viral nucleic acid which does not overlap with the first region. In yet another embodiment, the pharmaceutical formulation includes an orally tolerable carrier.

A method of increasing the cellular uptake and intracellular concentration of an exogenous oligonucleotide is also provided by the present invention. In this method, a cell is treated or contacted with the pharmaceutical formulation described above. Once inside the cell a cellular enzyme cleaves the lipophilic group on the prodrug from the reversibly derivatized nucleotide, thereby regenerating the parent oligonucleotide from the oligonucleotide prodrug. In this way, the intracellular concentration of the oligonucleotide is increased. In some preferred embodiments, the lipophilic group is cleavable by an esterase or phosphoramidase.

In another aspect of the invention, a method of treating a cell for viral infection, or of preventing viral infection in the cell, is

provided. In this method, the cell is contacted with an oligonucleotide prodrug having a nucleotide sequence complementary to a portion of the nucleic acid of a virus. The oligonucleotide prodrug enters the cell wherein an esterase or phosphoramidase cleaves the lipophilic chemical group from the derivatized nucleotide, thereby releasing the parent oligonucleotide. The oligonucleotide then hybridizes to a complementary portion of the viral nucleic acid. Thus, the invention provides a useful composition for treating inadvertently infected cell culture lines. Contamination of cell lines with viruses or mycoplasma can be eliminated by using the compositions according to the invention.

In yet another aspect, the invention provides a method of increasing the intracellular or *in vivo* lipid solubility and bioavailability of an oligonucleotide. In this method an oligonucleotide is derivatized to form an oligonucleotide prodrug which is more lipid soluble and bioavailable than the oligonucleotide. As described above, the prodrug includes at least six covalently linked nucleotides, at least one of which nucleotide has a 5' phosphate, a 3' phosphate, or an internucleotidic phosphate linkage to which is reversibly and covalently attached a lipophilic chemical group, and which is cleavable with a cellular esterase or a phosphoramidase.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other aspects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a diagrammatic representation of a generalized scheme depicting the conversion of an oligonucleotide prodrug to an oligonucleotide, wherein "Z" is a functional group, "X" is O, S, or NR (R is alkyl or aryl), Y is O or S, R is alkyl, aryl, ar-alkyl, heterocyclic group, fatty acid, or steroid, " R" " is a branching group, and "Q" is a heteroatom such as O or S, or is a covalent bond;

FIG. 2 is diagrammatic representation of the action of an enzyme on various lipophilic groups of different oligonucleotide prodrugs to yield the same parent oligonucleotide;

FIG. 3 shows a diagrammatic representation of the general structure of a steroid nucleus which can be covalently attached to a nucleotide via any site on the steroid via a Z (amide or ester) group;

FIG. 4 is a schematic representation of the bioactivation of acyloxyalkyl ester-type prodrug 1 with esterases to yield oligonucleotide 5;

FIG. 5 is a diagrammatic representation of the preparation of iodoalkylacylates **10a-d** and the treatment of R_p **2** or S_p **2** with iodoalkylacylates **10a-d** to yield the S-alkyl dinucleoside, phosphorothioates **3a-3d**;

FIG. 6 is a schematic representation of the hydrolysis of d(TpsT) esters **3a-c** to yield compound **4** and parent oligonucleotide **2**;

FIG. 7A is a collection of reversed-phase HPLC profiles of the time course of hydrolysis of R_p **3b** with human serum, wherein the arrows indicate the retention times in minutes;

FIG. 7B is a collection of reversed phase HPLC profiles of the time course of hydrolysis of S_p **3b** with human serum, wherein the arrors indicate the retention times in minutes;

FIG. 8 shows the ^{31}P-NMR spectra and autoradiogram of a polyacrylamide gel of a PS/PO containing parent oligonucleotide (A), the oligonucleotide prodrug (B), and the oligonucleotide prodrug after incubation with an esterase for 24 hours (C); and

FIG. 9 is an autoradiogram of parent oligonucleotide (lanes 1 and 3), prodrug oligonucleotide (lanes 2 and 4), and prodrug oligonucleotide after incubation with an esterase for 36 hours.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patents, allowed applications, and references cited herein are hereby incorporated by reference.

In order for antisense oligonucleotides to elicit their therapeutic action as inhibitors of gene expression, they must be taken up by cells and internalized. However, if the oligonucleotide is polyionic and of high molecular weight, its ability to cross lipid membranes is reduced; oligonucleotides that carry less negative charges are known to be taken up by cells more efficiently (Temsamani et al. (1994) *Antisense Res. Dev.* 4:35-42).

The present invention provides a method of improving oligonucleotide uptake through lipid membranes into cells, thereby increasing the efficacy of treatment and reducing the dose of antisense oligonucleotide required. In this approach, oligonucleotide-containing prodrugs have been designed which undergo an enzyme-mediated transformation near or within the target organ, tissue, or cell to release the functional parent antisense oligonucleotide. The oligonucleotide prodrugs are oligonucleotides that have been reversibly derivatized to become lipophilic, less ionic oligonucleotide conjugates having the ability to enter cells by passive diffusion through cell membranes and also to get transported

across various physiologic barriers including the blood-brain barrier.

The oligonucleotide prodrugs include at least six, and preferably 10 to 30 nucleotides. The 3' terminus of one nucleotide is covalently linked to the 5' terminus of the next nucleotide. The nucleotides may be deoxyribonucleotides or analogs thereof, ribonucleotides or analogs thereof, or a combination of deoxyribonucleotides, deoxyribonucleotide analogs, ribonucleotides, and ribonucleotide analogs, thereby forming a chimeric oligonucleotide prodrug.

The term "nucleotide analog" as used herein encompasses a nucleotide not found naturally *in vivo* and having a synthetic group attached or replacing its 3' or 5' terminal chemical groups. Thus a nucleotide analog forms an internucleotide linkage other than a phosphodiester between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphate has been replaced with any number of chemical groups. Preferable synthetic linkages include alkylphosphonates, phosphate esters, alkylphosphonates, phosphorothioates, phosphorodithioates, carbonates, alkylphosphonothioates, phosphoramidates, carbamates, phosphate triesters, acetamidate, and carboxymethyl esters.

The term "nucleotide analog" also encompasses nucleotides with a modified base and/or sugar. For example, a 3', 5'-substituted nucleotide is a

modified nucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). A modified nucleotide may also be a capped species. In addition, unoxidized or partially oxidized nucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides. Also considered as modified nucleotides are those having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found *in vivo* without human intervention. Modifications may also include a substitution at the phosphate group. For example, the oxygen at the 5' phosphate group may be substituted with a sulfur, amine, or other group. Also considered as modified nucleotides are nucleotides having various other structural modifications not found *in vivo* without human intervention.

At least one nucleotide of the oligonucleotide prodrug has been derivatized such that the prodrug becomes less ionic and more lipophilic than it was before derivatization. This is accomplished by covalently attaching a lipophilic chemical group to the 3' phosphate, 5' phosphate, or internucleotidic phosphate group of the nucleotide at a sulfur, oxygen, or amine group, shown as prodrug **1** in FIG. 1. Some preferred nucleotides to which the lipophilic chemical group can be attached include

phosphorothioates, phosphorodithioates, phosphoramidates, and phosphate esters.

At least one nucleotide of the prodrug is derivatized as described above, and all of the nucleotides may be likewise derivatized. The derivatized nucleotides may be located anywhere in the oligonucleotide prodrug, i.e., they may be in the internal or terminal regions of the prodrug, or may be scattered throughout the molecule.

TABLE 1 below lists some representative oligonucleotide prodrugs having 6, 17, 25, and 30 nucleotides. "↓" indicates the position of the derivatized nucleotide residue.

TABLE 1A

OLIGONUCLEOTIDE PRODRUG SEQUENCE	SEQ ID NO.
AAA'TGT	3
A'AATGT	3
AA'ATGT	3
A'A'A'T'G'T	3
CGG'CAA	4
C'GGCAA	4
CG'G'CAA	4
C'G'G'C'A'A	4
UGC'CAG	5
U'GCCAG	5
UG'CC'AG	5
U'G'C'C'A'G	5
GTAAAACGACGGCCAG'T	6
GTAAAACG'ACGGCCAGT	6
G'TAAAACGACGGCCAG'T	6
G'T'A'A'A'A'C'G'A'C'G'G'C'C'A'G'T	6
GTATTCAAAGGAGTAC'C	7
GTATTCAA'A'GGAGTACC	7
G'TATTCA'AAGGA'GTAC'C	7
G'T'A'T'T'C'A'A'A'G'G'A'G'T'A'C'C	7
GAGCAUCACGGUGAGC'G	8
GAGCAUCA'C'GGUGAGCG	8
G'AGCA'UCACGGUG'AGC'G	8
G'A'G'C'A'U'C'A'C'G'G'U'G'A'G'C'G	8

TABLE 1B

OLIGONUCLEOTIDE PRODRUG SEQUENCE	SEQ ID NO:
C'UCUCGGACCCATCTCTCTCCUUCU	2
CUCUCGGA'CCCATCTC'TCTCCUUCU	2
CUCUC'G'G'ACCCATCT'CTCTCCUU'C'U	2
C'U'C'U'C'G'G'A'C'C'C'A'T'C'T'C'T'C'T'C'C'U'U'C'U	2
C'TCTCGGACCCATCTCTCTCCTTCT	1
CTCTCGGA'CCCATCTC'TCTCCTTCT	1
CTCTC'G'G'ACCCATCT'CTCTCCTT'C'T	1
C'T'C'T'C'G'G'A'C'C'C'A'T'C'T'C'T'C'T'C'C'T'T'C'T	1
G'AATGACTGATTGAGTGACTGAATGCCCGT	9
GAATGACTGATTGAGTGACTGAATGCC'CG'T	9
GAATGA'C'TGATTGA'GTGACTGAA'TGCCCGT	9
G'A'A'T'G'A'C'T'G'A'T'T'G'A'G'T'G'A'C'T'G'A'A'T'G'C'C'C'G'T	9
C'AGUGACUGACUGAGCGACUGAACUCCCGT	10
CAGUGACUGACUGAG'CGACUGAACUCCCG'T	10
CAGU'G'ACUGA'C'UGAGCG'ACUGAACUC'CCGT	10
C'A'G'U'G'A'C'U'G'A'C'U'G'A'G'C'G'A'C'U'G'A'A'C'U'C'C'C'G'T	10

The derivatizing chemical group may be any chemical group which is lipophilic and which decreases the ionic strength of the oligonucleotide as a whole. Useful lipophilic chemical groups include, but are not limited to, alkyl, aryl, ar-alkyl, alkane groups. Other useful lipophilic groups include fatty acids or carboxylic acids with long hydrocarbon chains having from about 3 to about 40 carbons, and heterocyclic compounds having a 5 or 6 membered carbon ring or a fused polycyclic system containing heteroatoms such as O, N, S, or P at one or multiple positions in the system. Non-limiting examples of heterocyclic compounds include thiophene, imidazole, pyrimidine, pyrrole, furan, and purines, and steroids such as steroid esters and steroid amides. Yet other useful lipophilic groups are steroids have from about 17 to about 40 carbons, and preferably from about 17 to about 32 carbons. FIG. 3 shows the general structure of a steroid having 4 carbon rings and 17 positions at which the lipophilic group may be attached. The lipophilic chemical groups attached to multiple derivatized nucleotides may be the same or different.

The sequence of the nucleotides in the oligonucleotide prodrugs of the invention may be any sequence, as the ability of the oligonucleotide prodrug to pass or be transported through cell membranes is not sequence-dependent. Thus, the sequence of nucleotides in the oligonucleotide prodrugs may vary according to the

purpose for which the antisense oligonucleotide is being used. For example, if the oligonucleotide is being used to prevent or treat a specific viral infection, at least a portion of the nucleotide sequence of the prodrug will be complementary to a portion of the nucleotide sequence of the viral nucleic acid. Alternatively, the antisense oligonucleotide may be used to control the expression of a particular gene encoding a protein of interest in the target cell or tissue, such as an enzyme. The nucleotide sequences of many viruses and cellular genes are known and antisense oligonucleotides have been prepared thereto.

The oligonucleotide prodrugs of the invention are prepared by synthesizing the antisense oligonucleotide using nucleotides capable of derivatization, and then derivitizing or covalently linking the lipophilic chemical group to a reactive group on the oligonucleotide.

The parent antisense oligonucleotide of the invention can be prepared by any art recognized method (reviewed in *Protocols For Oligonucleotides and Analogs* (*Meth. Mol. Bio.* (Agrawal, ed.) Humana Press, Totowa, NJ, Volume 20, 1993); Goodchild (1990) *Bioconjugate Chem.* 1:165-187; and Uhlmann et al. (1990) *Chem. Rev.* 90:543-584). For example, nucleotides can be covalently linked using techniques such as phosphoramidate, H-phosphonate chemistry, methylphosphoramidate, or methoxy-phosphoramidite chemistry which can be carried out manually or by an automated synthesizer and then processed.

The oligonucleotides of the invention may also be modified in a number of ways without compromising their ability to be derivatized or to hybridize to a target nucleic acid. For example, modifications include those which are internal or are at the end(s) of the oligonucleotide molecule and include additions to the molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s) or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule (reviewed in Agrawal et al. (1992) *Trends Biotechnol.* 10:152-158).

The nucleotide in the oligonucleotide prodrug is derivatized with a lipophilic chemical group attached to the 3' phosphate, 5' phosphate, or

internucleotide phosphate group of at least one nucleotide in the oligonucleotide. Covalent linkage of the chemical group can be accomplished by any art recognized protocol specific for the group to be appended such as an amide or ester.

Once inside the cell, target tissue, or body in general, the oligonucleotide prodrug is processed by an endogenous enzyme such as esterase or phosphoramidase. This enzyme may be tissue- or cell-specific, and thus the oligonucleotide prodrug may be designed such that the lipophilic chemical groups are cleaved from the drug, thereby regenerating the parent antisense oligonucleotide only when the prodrug reaches or approaches the target tissue or cell. FIG. 1 depicts the generalized scheme of parent oligonucleotide (compound 5) regeneration from prodrug 1 with an enzyme, and FIG. 2 illustrates the specific action of an enzyme on various specific lipophilic chemical groups attached to the oligonucleotide prodrug.

Enzymes which release the lipophilic group from the oligonucleotide include esterases and phosphoramidases. Useful esterases found in cells and body tissues include but are not limited to thiol proteases, carboxyl proteases, metalloproteases, and serine proteases such as trypsin, chymotrypsin and elastase (found in the pancreas), thrombin, plasmin, and complement C1 (found in the serum), kallikrein (found in the blood and tissues), acrosomal protease (found in

sperm), and lysosomal protease (found generally in animal cells).

For example, FIG. 4 shows the regeneration of an antisense oligonucleotide phosphorothioate, phosphorodithioate, or phosphoramidate from a prodrug form of the oligonucleotide (prodrug 1) where X=S. In prodrug 1, a labile carboxylic ester group has been incorporated so that an enzyme-mediated hydrolytic attack is directed to a highly electrophilic carbonyl carbon center rather than to the phosphoryl group of the phosphorothioate. This, in turn, ensures the regeneration of the phosphorothioate rather than the native phosphodiester backbone during the bio-activation (i.e., regeneration of the parent antisense oligonucleotide) *in vivo*. An acyloxyalkyl ester type group fulfills the requirements of an ideal appendage for the phosphorothioate oligonucleotides. Thus, the acyloxyalkyl ester type prodrug 1 undergoes bio-activation with an esterase to give the unstable hydroxymethyl oligonucleotide 1a which then readily eliminates formaldehyde to give the parent phosphorothioate oligonucleotide 5. The rate of enzymatic hydrolysis is modulated by choosing various acyl groups, with the more hindered derivatives such as prodrug 1 (where R = t-butyl) undergoing slower hydrolysis.

Incorporation of the acyloxyalkyl as well as aryl, alkyl, ar-alkyl, heterocyclic, fatty acid, steroid esters, and steroid amide groups into phosphorothioates, phosphorodithioates or

phosphoramidates results in lipophilic and less ionic oligonucleotides. Such modifications enable these prodrugs to be efficiently taken up by cells, where cellular esterases or phosphoramidases hydrolyze the ester or amide group in the prodrug to regenerate the parent oligonucleotide.

The following methodology was designed for the chemoselective S-functionalization of an dinucleotide phosphorothioate as a model for the preparation of various oligonucleotide prodrugs. The dinucleoside phosphorothioate 2 [d(TpsT)] bearing the 5' dimethoxytrityl group (DMT) at the 5' end was synthesized on a 10 x 10 μmole scale using known phosphoramidite chemistry on an automated DNA synthesizer (see, e.g., Beaucage et al. (1992) *Tetrahedron* **48**:2223-2311). Oxidative sulfurization of the internucleotidic phosphite linkage was carried out using 3H-1,2-benzodithiole-3-one-1,1-dioxide to generate the phosphorothioate linkage as described by Iyer et al. (*J. Org. Chem.* (1990) **55**:4693-4698 and *J. Am. Chem Soc.* (1990) **112**:1253-1254). Following the synthesis, the controlled-pore-glass (CPG) support was treated with 28-30% NH_3 to cleave the dinucleoside phosphorothioate from the support and remove the β-cyanoethyl phosphate protecting group. The dimer was then subjected to reverse-phase HPLC to isolate the constituent R_p (retention time (R_t) = 39 min.) and S_p (R_t, 35 min.) diastereomers of phosphorothioate prodrug 2 bearing the 5'-DMT group. Each of the individual R_p and S_p diastereomers were then treated with 80%

acetic acid to remove the 5'-DMT group and purified again by reverse phase HPLC to obtain pure R_p and S_p isomers (R_p:R_t, 24.2 min.; S_p:R_t 25.4 min.).

The assignment of absolute configurations "R_p" and "S_p" to the individual diastereomers of prodrug **2** follows the well-established literature precedent (Connolly et al. (1984) *Biochem.* **23**:3443-3453), and is based on the relative mobilities of the R_p and S_p dinucleoside phosphorothioates (5'-DMT "on" and 5'-DMT "off") on reverse-phase HPLC.

To further confirm these assignments, the individual diastereomers of PS-prodrug **2** were treated with snake venom phosphodiesterase (type II) using the method of Connolly et al. (*ibid.*). The snake venom stereospecifically hydrolyzed the R_p diastereomer (R_t, 24.2 min., δ 52.6 ppm) and nuclease P1 which hydrolyzed the S_p diastereomer (R_t, 25.4 min., δ 52.2 ppm).

The diastereomers of prodrug **2** were converted to the S-alkyl phosphorothioates (PS-prodrugs **3a-d**) using a chemoselective S-alkylation protocol (e.g., Agrawal et al. (1991) *Nucl. Acids Res.* **18**:5419-5423), as shown in FIG. 5. The iodoalkylacylates (compounds **10a-d**) required for the alkylation reactions were prepared from the corresponding chloroalkylacylates (FIG. 4) using the chloroalkylacylates according to the method of Iyer et al. (*Tetrahedron Lett.* (1989) **30**:7141-7144). These, in turn, were synthesized by the reaction of the corresponding acid chlorides with

paraformaldehyde in presence of catalytic amounts of anhydrous zinc chloride, as described by Ulich et al. (*J. Am. Chem. Soc.* (1921) 43:660). The reactions were monitored by reverse-phase HPLC, and no evidence of any epimerization at the chiral phosphorous center was noted. Significant side products were not detected reflecting the lack of reactions at other sites. Thus, R_p2 gives R_p 3a-d, and S_p 2 gives S_p 3a-d.

In all cases, the reaction mixture was worked up and products isolated by preparative reverse-phase HPLC. The retention times of the various analogs of prodrug 3 are shown in TABLE 2.

TABLE 2

Buffer Hydrolysis of 3a-c

Compound	R_t (min)	$t_{1/2}$(days)	% parent (analog 2)
R_p 3a	38.7	>5	22
S_p 3a	39.3	>5	20
R_p 3b	41.6	>10	25
S_p 3b	42.2	>10	25
R_p 3c	44.3	>30	46
S_p 3c	44.9	>30	35

As measured by ^{31}P-NMR, the R_p isomer of analog 3c typically had a value of 24.8 ppm and the S_p isomer of 3c had a δ value 25.8 ppm. The

R_p and S_p triesters of 3, unlike the R_p and S_p diester counterparts (i.e., analog 2) were resistant to hydrolysis by snake venom phosphodiesterase and P1 nuclease, respectively. These results indicate that the prodrug is less susceptible to nuclease digestion than is its parent.

Although prodrugs 3a-d are phosphotriesters bearing a labile carboxylic ester moiety, they are easily isolated and purified; they are soluble in aqueous buffers and in organic solvents such as acetonitrile and chloroform. They can be stored indefinitely in aqueous buffers (pH 7.0) at 0-5°C with no evidence of decomposition. However, upon prolonged storage in aqueous buffers (pH 7.0) at ambient temperature, some decomposition occurs. TABLE 2 above shows the half-lives of decomposition of the analogs in aqueous buffers at ambient temperature. As would be expected, the less hindered analogs 3a were more susceptible to hydrolytic decomposition than the more hindered analogs 3b-c. The major product of decomposition was the desulfurized product, the natural diester, 4.

Prodrug analogs 3a-3c were then analyzed for their ability to undergo hydrolysis in serum. These serum-mediated hydrolysis studies were carried out on HPLC-purified materials containing ammonium acetate (i.e., salt). To determine whether the presence of salt had an impact on the kinetics and product profile of hydrolysis, (i.e., on bio-reversibility), esters 3a-c (HPLC mobile

phase containing or not containing salt were incubated with human serum. TABLE 3 shows the half-lives ($t_{1/2}$) of hydrolysis of analogs 3a-c in the presence and absence of salt.

TABLE 3

Hydrolysis of Analogs 3a-c by Human Serum

Compound	R_t (min)	$t_{1/2}$ (min)	k (x $10^{-2}min^{-1}$)	% compound 2
R_p3a	38.7	40	1.73	99(R_p)
S_p3a	39.3	11	6.30	99(S_p)
R_p3b	41.6	82	0.85	99(R_p)
S_p3b	42.2	28	2.47	99(S_p)
R_p3c	44.3	1980	0.04	95(R_p)
S_p3c	44.9	335	0.21	95(S_p)
R_p3a*	38.7	13	n.d.	98(R_p)
S_p3a*	39.3	4.0	n.d.	99(S_p)
R_p3b*	41.6	23	n.d.	97(R_p)
S_p3b*	42.2	5.0	n.d.	99(S_p)
R_p3c*	44.3	163	n.d.	75(R_p)
S_p3c*	44.9	68	n.d.	87(S_p)

@ estimated at $t_{1/2}$ of hydrolysis

* no salt

n.d. not determined

In all cases, stereospecific hydrolytic conversion of the S_p triesters 3a-c to the R_p 2 was observed. The S_p esters 3a-c were hydrolyzed much faster compared to the R_p esters 3a-c. In addition, the formation of significant amounts of phosphoric diester 4 as a by-product was seen upon hydrolysis of 3c.

As TABLE 3 shows, when the same serum-mediated hydrolysis studies were done using salt-free materials, the half-lives of hydrolysis were significantly reduced. Typically, the half-life of hydrolysis R_p 3c* (salt free) was 163 minutes, whereas that of R_p 3c (with salt) was 1,980 minutes. Increased formation of the desulfurized products 4 was also observed, especially in case of the hindered analogs R_p and S_p 3c, when the enzyme-mediated hydrolysis was carried out in the absence of salt. In that event, the origin of the desulfurized product 4 in the case of analogs 3a-c, is likely to follow the path shown in FIG. 6.

Alternatively, at least part of the desulfurized product may be formed due to hydrolysis mediated by a phosphodiesterase-like activity present in serum, and that ammonium acetate may suppress this phosphodiesterase-like activity and reduce esterase activity.

Studies were thus undertaken to confirm that the factor(s) present in serum which is responsible for mediating the hydrolysis of the esters has esterase-like activity. In these only salt-free materials were used. Porcine liver

esterase (which is a mixture of at least seven enzymes) was used as a typical carboxyl esterase enzyme. The reactions were monitored by reverse-phase HPLC using a gradient of 100% ammonium acetate buffer (0.1 M) to 80% acetonitrile in ammonium acetate (0.1 M). The date obtained from these studies was analyzed according to a first order kinetic model. The results are shown in FIGS. 7A and 7B, where the arrows indicate the retention times in minutes of **4**, **2**, and **3b**, and are summarized in TABLE 4.

TABLE 4

Hydrolysis of **3a-c** (Salt-Free) by Pig Liver Carboxy Esterase

Compound	R_t (min)	$t_{1/2}$ (min)	% compound 2°
R_p**3a**	38.7	nd	n.d.
S_p**3b**	39.3	nd	n.d.
R_p**3b**	41.6	42	99 (R_p)
S_p**3b**	42.2	64	99 (S_p)
R_p**3c**	44.3	185	97 (R_p)
S_p**3c**	44.9	430	98 (S_p)

@ estimated at $t_{1/2}$ of hydrolysis, remaining being **4**.

n.d. not determined

Upon incubation of the substrates 3a-c with pig liver esterase in a stoichiometric ratio (one unit/one μmole of substrate), almost instantaneous stereospecific hydrolysis was observed to give the desired product R_p or S_p 2. Under these conditions, no stereo-differentiation in the rates of hydrolysis of 3a-c was noted (i.e., both R_p and S_p were hydrolyzed at the same rate). Also, no difference in the half-lives of the hindered and less hindered analogs was noted. These observations reflect a high binding affinity of the substrate for the enzyme and a fast catalyst rate. However, upon lowering the enzyme concentration, some stereo-differentiation was noted as previously observed in case of the serum studies. Inverse stereochemical preference was observed; R_p was hydrolyzed slightly faster (R_p 3c, $t_{1/2}$ = 185 min.) than S_p (S_p 3c, $t_{1/2}$ = 430 min.). These results suggest that pig liver esterases have different stereochemical specificities for substrates 3a-c when compared to human plasma carboxyl esterase. As in case of the serum studies, the more hindered t-butyl analogs were hydrolyzed more slowly compared to the less hindered analogs. The formation of the desulfurized product 4 was also observed, especially in the case of hindered analogs 3c, under these conditions as in case of the serum-mediated hydrolysis studies.

In order to get further insight into the mechanism of the hydrolysis reaction and to demonstrate that hydrolysis proceeds by initial attack on the carboxyl group, the R_p and S_p

benzoyl analog **3d** was prepared. Incubation of R_p or S_p **3d** with pig liver esterase gave analog **2** along with the formation of benzoic acid which was identified by co-chromatographic comparison with an authentic standard. These data are indicative of an initial nucleophilic attack on the carbonyl carbon by esterases, rather than attack at the phosphoryl group, to generate analog **2**. A pathway for the formation of analog **4** is shown in FIG. 6. This pathway involves an initial nucleophilic attack by the serine hydroxyl group of the esterase on the ester carbonyl center to generate the oxy-anion intermediate **9** which performs an intramolecular attack on the juxta-positioned phosphorous center to give cyclic intermediate **11**. Fragmentation of intermediate **11** by *path a* gives the desired product **2**, whereas fragmentation of **11** by *path b* gives the desulfurized product **4**, each pathway generating the same acyl-enzyme intermediate **12**. The enzyme-mediated hydrolysis gives the expected phosphorothioate **2** (by *path a*) as the predominant product.

Based on the results of the model described above, an oligonucleotide prodrug and its parent oligonucleotide, both having SEQ ID NO:2, were examined before and after hydrolysis with an esterase by NMR spectroscopy and polyacrylamide gel electrophoresis. The oligonucleotides were dissolved in D_2O, and the NMR spectra recorded. The results are shown in FIG. 8 wherein A is the spectrum of the parent oligonucleotide, B is the spectrum of the prodrug, and C is the spectrum of

the prodrug that had been incubated with an esterase.

As shown in FIG. 8, a chemical shift is seen in the spectrum of the phosphorus nuclei which have been derivatized with a lipophilic group (in A it is at about δ 57, wherein in B, it has shifted to the right). Furthermore, a shift in the spectrum of the derivatized phosphorus nuclei back to the position it was at in the parent after 24 hours of esterase digestion (δ-8) is seen in C, demonstrating the reversibility of the derivatization.

The species analyzed in A, B, and C were subjected to polyacrylamide gel electrophoresis and autoradiography, as shown in FIGS. 8 and 9. These autoradiograms demonstrate that the oligonucleotide prodrug is converted back to the parent oligonucleotide within 36 hours of incubation with the enzyme.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Synthesis of d(TpsT) and Parent Oligonucleotides

The automated solid-phase synthesis of d(TpsT) 2 and oligonucleotides was carried out on a 10 x 10 μmole scale on a DNA synthesizer (Biosearch 8700, Bedford, MA using phosphoramidite chemistry (Beaucage et al. (1992) *Tetrahedron* 48:2223-2311). The oxidative sulfurization reaction required for the preparation of oligodeoxyribonucleoside phosphorothioates was effected by a 0.2 M solution of crystalline. 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (R.I. Chemical Co., Costa Mesa, CA), in acetonitrile as described by Iyer et al. (*J. Am. Chem. Soc.* (1990) 112:1253-54; *J. Org. Chem.* (1990) 55:4693-98). The sulfurization reaction was performed over a period of 45 seconds to 2 minutes depending on the scale of synthesis. Following synthesis, the controlled pore glass (CPG) support was treated with 28-30% NH_3 at 55°C for 8-10 hours to cleave the dinucleoside phosphorothioate from the support and remove the β-cyanoethyl phosphate protecting group. The R_p:S_p ratio of 2 was estimated to be 60:40 based on ^{31}P-NMR and HPLC analysis.

2. Synthesis of Iodoalkyl Acylates

Iodoalkyl acylates **10a-d** were prepared and characterized as previously described by Srivastva et al. (*Bioorg. Chem.* (1984) 12:118-129), and by Iyer et al. (*Tetrahedron. Lett. (1989)* 30:7141-7144).

Briefly, to a 117 mM solution of sodium iodide (17.56) in 100 ml dry acetonitrile, was added 12.70 g (85 mM) chloroalkyl acylate over a period of 30 minutes at 25°C in the dark. A white precipitate of NaCl began to appear immediately. The contents were stirred for 12 hours. The precipitate was filtered, and the acetonitrile was removed from the filtrate *in vacuo*. The filtrate was taken up in 70 ml toluene, washed two times with 40 ml 5% aqueous sodium bisulfite, and then 40 ml water. The toluene layer was then dried over anhydrous sodium sulfate. Toluene was removed *in vacuo* and distillation of the resulting pale yellow oil gave a clear, colorless liquid (48-50°C, 3 mm Hg, 14.2 g, 70%) ^{1}H-NMR ($CDCl_3$) δ ppm 1.19 (s, 9H), 5.91 (s, 2H) ^{13}C-NMR ($CDCl_3$): δ ppm 26.4 (CH_3), 3L4 (CH_2), 38.7 (-C), 176.0 (CO)). The distilled products **10a-d** were stored at -80°C until ready to use.

3. Synthesis of Dinucleoside S-alkyl Phosphorothiolates

The esters **3a-c** were synthesized by reacting 50 A_{260} units of R_p or S_p **2** in (0.5 ml 250 mM Tris buffer, pH 7.0) with the corresponding iodoalkyl acylates **10a-d** (2 mmoles) in 3 ml acetonitrile, at 37°C for 3-4 hr. The reaction mixture was quenched with 100 µl 0.5% sodium bisulfite, evaporated to dryness *in vacuo* and subjected to preparative reverse-phase HPLC as described below. The solvent was removed *in vacuo* and the esters (**3a-c**) thus obtained (isolated yields 60-70% based on

compound 2), were used as such for further studies. NMR spectra were recorded on a spectrometer operating in the presence of broad band decoupling at 7.05 Tesla (300 MHz for ^1H. ^{31}P-NMR spectra were recorded in deuterated solvents using trimethylphosphate as the external reference. Typical ^{31}P-NMR (D_2O): δ R_p 3c, 24.8; Sp 3c 25.8 *ppm*.

4. · Preparative HPLC

Deprotected TpsT dimer bearing the DMT group at the 5'-end was purified by reverse-phase HPLC using a C-18 reverse-phase column (125Å, 55-105 μM, WATERS (Milford, MA), and a gradient of 100% A to 100% B over 70 minutes [A: $CH_3CO_2NH_4$ (0.1 M in water); B: acetonitrile: $CH_3CO_2NH_4$ (0.1 M) (80:20)], using a flow rate of 12 ml/min. The TpsT DMT-on peaks (R_t = 41 and 45 min) were collected and subjected to detritylation using 80% acetic acid for 30 min. The solvent was removed and the crude compound 2 subjected to reverse-phase HPLC as described below using a C-18 column developed with a gradient of 100% A to 100% B over 70 min, using either A, (0.1 M $CH_3CO_2NH_4$ in water); B (acetonitrile : 0.1 M $CH_3CO_2NH_4$, *80:20) or A (water) and B (acetonitrile : water (80:20)). Use of the latter system afforded salt-free materials. The R_p and S_p 2 fractions were collected, evaporated, lyophilized and stored at 0°C until ready to use. ^{31}P-NMR (D_2O) : δ R_p 2, 52.6 and S_p 2, δ 52.2 ppm.

5. Bioreversibility Studies

A. Hydrolysis With Snake Venom Phosphodiesterase.

To confirm the R_p and R_s assignments, the individual diastereomers of 2 were treated with snake venom phosphodiesterase (type II) which stereospecifically hydrolyzed the R_p diastereomer (R_t = 24.2 min., δ = 52.6 ppm) and nuclease P1 which hydrolyzed the S_p diastereomer (R_t = 25.4 min., δ = 52.2 ppm). This was accomplished by using the method of Connolly et al. (*Biochem.* (1984) 23:3443-3453). Snake venom phosphodiesterase was obtained from Boehringer Mannheim GmbH, Indianapolis, Indiana, in a suspension of 50% glycerol, pH 6.0.

B. Hydrolysis With Buffer

The hydrolysis mixture contained about. 0.6 A_{260} units of substrates 3a-3c in 80 μl 25 mM Tris buffer, pH 7.0 at 37°C. At each time point, 10 μl aliquots of incubation mixture were diluted with 140 μl buffer A and analyzed by reverse-phase HPLC, (600E instrument, Waters, Milford, MA) using a C18 4μ Radial Pak cartridge column (Waters, Milford, MA), developed with a gradient (100% A to 60% B over 60 minutes) of buffer A (0.1 M $CH_3CO_2NH_4$)) and buffer B (80:20, CH_3CN:0.1 M $CH_3CO_2NH_4$), with a flow rate 1.5 ml/min. Retention times (R_t) of R_p 2, were 24.2; S_p 2, 25.4; and 4, 21.0 minutes respectively. Prodrugs 3a-3c were

converted back to the starting dinucleotides after exposure to buffer.

C. Serum Hydrolysis

The hydrolysis mixture contained about 0.6 A_{260} units of substrates 3a-3c, 20 µl human serum (GIBCO, BRL, Gaithersburg, MD) in 60 µl of 25 mM Tris buffer, pH 7.0 at 37°C. At each time point, aliquots of incubation mixture were diluted with 140 µl buffer A and analyzed by reverse-phase HPLC, as described in EXAMPLE 5B above. Prodrugs 3a-3c were converted back to the starting dinucleotides after exposure to serum, as shown in FIG. 7A and 7B.

D. Hydrolysis With Porcine Liver Esterase

The hydrolysis mixture contained about 0.6 A_{260} units of substrates 3a-3c and µl of pig liver carboxyl esterase in 60 µl of 25 mM Tris buffer, pH 7.0) at 37°C. At each time point, 10 µl aliquots of incubation mixture were diluted with 140 µl buffer A and analyzed by reverse-phase HPLC, as described in EXAMPLE 5B above. Prodrugs 3a-3c were converted back to the starting dinucleotides after exposure to the pig liver esterase.

6. Preparation of Oligonucleotide Prodrugs

To a solution of 90 O.D. of an oligonucleotide (SEQ ID NO:1 or SEQ ID NO:2) in 0.5 ml 250 mM Tris-HCl buffer, pH 7.0, was added

20 μl iodomethyl isobutyrate in 0.5 ml acetonitrile. The solution was incubated at 37°C for 1-3 hr. The pH of the solution was maintained around 6-7 by adding trace amounts of triethyl amine periodically. At the end of the reaction, the solvent was removed under pressure and the residue dissolved in 200-500 μl water and 30-40 μl 1 M sodium chloride solution. To the solution was added 1-1.2 ml cold ethanol, and the solution kept at about -80°C for 1-2 hr. The solution was centrifuged at 10,000 g for 15 minutes, and the resulting pellet analyzed by HPLC and gel elctrophoresis, or dissolved in sodium chloride solution and ethanol precipitated as above.

As shown in FIG. 8, ^{31}P-NMR of this product showed a signal at δ 25 ppm as compared to the starting oligonucleotide at δ 51 ppm. Analysis by gel electrophoresis (20% polyacrylamide) of the product showed a slow moving band as compared to the starting oligonucleotide.

7. Bio-reversibility Studies with Oligonucleotide Prodrugs

A. Hydrolysis with Pig Liver Esterase

To 1.5 A_{260} units of prodrug (in 25 μl 250 mM Tris, pH 7.2) was added 2 μl pig liver esterase and the reaction mixture incubated at 37°C overnight. Aliquots of the reaction mixture were then analyzed by gel electrophoresis using a 20% polyacrylamide, 7 M urea denaturing gel. FIGS. 8 and 9 show the profile of the reaction mixture

obtained after 24 and 36 hours, respectively. The prodrug oligonucleotide is converted back to the parent oligonucleotide after exposure to the pig liver esterase.

B. Hydrolysis with Serum

To 1.5 A_{260} units of the prodrug (25 μl) oligonucleotide in 250 mM Tris buffer, pH 7.2) was added 40 μl human serum, and the reaction mixture incubated at 37°C overnight. Aliquots of the reaction mixture was analyzed by gel electrophoresis as described in EXAMPLE 7A. FIG. 8 shows the profile obtained of the incubate after 24 hours. The prodrug oligonucleotide is converted back to the starting oligonucleotide after exposure to serum.

C. *In vivo* Hydrolysis

Hybrid, chimeric prodrugs having SEQ ID NO:2 and a combination of 2-O-methyl ribonucleotides and phosphorothioates were administered in normal saline as a bolus intravenous injection into the tail vein of 150-200 g Sprague Dawley or albino rats. Three rats were used for each dose to provide doses of 1-10 mg/Kg. After administration, the animals were placed in metabolism cages and urine samples were collected for up to 72 hours. 0.25 ml blood samples are collected from the cut axilla region at period intervals following dosing. The samples were collected in microfuge tubes containing 0.25 μl of

27.5 mM EDTA at 0°C and centrifuged at 16,000 x g speed.

The plasma samples (150-200 μl) were analyzed by polyacrylamide gel electrophoresis (PAGE) in a 20% polyacrylamide, 7 M urea denaturing gel. These samples were also analyzed by HPLC to determine the half-life of bio-reversibility of the oligonucleotide prodrugs to parent oligonucleotide. The urine samples are also analyzed by PAGE and HPLC to determine content of the oligonucleotide prodrug and its metabolites. ^{35}S-labelled oligo-prodrugs are used in these studies.

Anti-HIV screening of the oligonucleotide prodrugs in chronically HIV-infected cells is conducted as described in Lisciewicz et al. (1993) *Proc. Natl. Acad. Sci. (USA)* 90:3860-3864.

These studies illustrate that the above-described specifically embodied oligonucleotide prodrugs of the invention, in addition to having favorable physicochemical and pharmacologic properties, have good therapeutic potential against AIDS.

8. Dinucleoside S-acyloxyaryl Phosphorothioate Prodrugs

In these prodrugs, generally depicted as indicated in Figure 2, the acyloxyaryl group is linked to the phosphorothioate group via a methylene bridge. Incorporation of the aryl group

in the appendage confers a certain degree of conformational rigidity to the appendage, providing greater stability of the prodrug in aqueous buffers within a wide pH range.

Acyloxyaryl phosphorothioate prodrugs were synthesized by reaching the underivatized phosphorothioate with 4-O-isobutyryl-α-iodotoluene, which was synthesized as described below.

Commercially available 4-hydroxybenzyl alcohol (Aldrich, Milwaukee, WI) was evaporated three times from pyridine, then dissolved in pyridine to yield 0.2 M 4-hydroxybenzyl alcohol. Chlorotrimethylsilan (Aldrich) was added in 1.2 molar equivalents, and the solution was stirred for fifteen minutes at room temperature. Isobutyryl chloride (Aldrich) was added in 1.2 molar equivalents, and the reaction was stirred for two hours. The reaction mixture was cooled to 0°C in an ice bath and excess water was added (50 equivalents). The ice bath was removed, and the reaction was stirred for four hours. The reaction mixture was concentrated and extracted with ethyl acetate. The organic layer was washed with 10% sodium bicarbonate solution. Evaporation yielded an oily residue which was purified by column chromatography on silica gel using hexane:ethyl acetate (80:20) as the eluent. Evaporation gave 4-O-isobutyryl benzyl alcohol as a colorless oil in 80-90% yield.

The 4-O-isobutyryl benzyl alcohol was then dissolved in a 1:2 mixture of ether:acetonitrile to a concentration of 0.28 M. Triphenyl phosphine, imidazole, and sublimed iodine were added in 1.5 molar equivalents each, and the reaction was stirred for two hours. The reaction mixture was extracted with ether. Concentration of the ether layer was followed by chromatography on a silica column, using hexane:ethyl acetate (90:10) as eluent. The fractions containing the product were concentrated to dryness and 4-O-isobutyryl-α-iodotoluene was obtained as a white solid in 90-95% yield.

4-O-isobutyryl-α-iodotoluene (27 mg) was dissolved in a 50:50 mixture of pH 7 Tris HCl buffer (0.5 M):acetonitrile. This solution was added to 30 O.D. units of TpsT and kept at 37°C for three hours. Every half hour, the pH was adjusted with triethylamine (Aldrich) to maintain pH = 6-7. After three hours, the reaction was complete as evaluated by HPLC.

The acyloxybenzyl dinucleoside phosphorothioate was obtained as an R_p, S_p mixture, which is a substrate for Porcine liver esterases (Sigma). Incubation of the acyloxybenzyl dinucleoside phosphorothioate with esterases resulted in rapid, stereospecific, and quantitative conversion to the parent phosphorothioate, with a quinomethide as a byproduct of the hydrolysis. The $t_{1/2}$ of the R_p acyloxybenzyl dinucleoside phosphorothioate was eight hours, and that of the S_p stereoisomer was

twelve hours. In addition, no desulfurized product resulted from hydrolysis of the prodrugs.

The half-lives of degradation of the prodrugs in buffers ranging from pH 2 to pH 8 was greater than 30 days at 22°C.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Hybridon, Inc.

(ii) TITLE OF INVENTION: Oligonucleotide Prodrugs

(iii) NUMBER OF SEQUENCES: 10

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Patentin Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
(B) REGISTRATION NUMBER: 33,523
(C) REFERENCE/DOCKET NUMBER: HYZ-025PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-330-1300
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CUCUCGCACC CATCTCTCTC CUUCU 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAATGT 6

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCAA 6

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

UGCCAG 6

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAAAACGAC GGCCAGT 17

(2) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTATTCAAAG GAGTACC 17

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGCAUCACG GUGAGCG 17

(2) INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATGACTGA TTGAGTGACT GAATGCCCGT 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA/mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CAGUGACUGA CUGAGCGACU GAACUCCCGT 30

What is claimed is:

1. An oligonucleotide prodrug comprising at least six covalently linked nucleotides, at least one nucleotide being derivatized with a lipophilic chemical group reversibly and covalently attached to the nucleotide at a 5' phosphate, a 3' phosphate, or an internucleotidic phosphate linkage,

the lipophilic group being selected from the group consisting of an ester or an amide, and the prodrug being reactive with a cellular or tissue enzyme which cleaves the lipophilic group from the derivatized nucleotide,

the enzyme being as esterase when the lipophilic group comprises an ester, and the enzyme being a phosphoramidase when the lipophilic group is an amide.

2. The oligonucleotide prodrug of claim 1 wherein the lipophilic chemical group is selected from the group consisting of an alkyl, aryl, alkane, ar-alkyl, heterocyclic group, fatty acid, steroid ester, steroid amide, and mixtures thereof.

3. The oligonucleotide prodrug of claim 1 wherein the lipophilic chemical group is attached to a sulfur, oxygen, or amine group on the nucleotide phosphate, the 5' phosphate,

4. The oligonucleotide prodrug of claim 1 wherein the nucleotide to which the lipophilic chemical group is attached is selected from the group consisting of a phosphorothioate, phosphorodithioate, phosphoramidate, and phosphate ester.

5. The oligonucleotide prodrug of claim 1 which is chimeric.

6. The oligonucleotide prodrug of claim 5 wherein at least one of the nucleotides is selected from the group consisting of a phosphorothioate, phosphorodithioate, phosphoramidate, phosphodiester, alkylphosphonate, alkylphosphonothioate, phosphoramidite, carbamate, carbonate, acetamidate, and carboxymethyl ester.

7. The oligonucleotide prodrug of claim 1 comprising at least one deoxyribonucleotide and at least one ribonucleotide.

8. The oligonucleotide prodrug of claim 7 wherein the ribonucleotide is a 2-O-alkyl ribonucleotide.

9. A pharmaceutical formulation comprising the oligonucleotide prodrug of claim 1.

10. The pharmaceutical formulation of claim 9 wherein the oligonucleotide prodrug comprises a nucleic acid sequence complementary to a region of a viral nucleic acid, and the formulation further comprises a second antiviral agent.

11. The pharmaceutical formulation of claim 10 wherein the oligonucleotide prodrug comprises nucleic acid sequence complementary to a first region of the viral nucleic acid, and the second antiviral agent is a second antisense oligonucleotide having a nucleotide sequence complementary to a second region of the viral which does not overlap with the first region.

12. The pharmaceutical formulation of claim 9 in a orally tolerable carrier.

13. A method of increasing the cellular uptake and intracellular concentration of an exogenous oligonucleotide,

the method comprising the step of treating a cell with a pharmaceutical formulation comprising the oligonucleotide prodrug of claim 1,

the enzyme cleaving the lipophilic group from the reversibly derivatized nucleotide, thereby regenerating the oligonucleotide from the oligonucleotide prodrug,

whereby the intracellular concentration of the oligonucleotide is increased.

14. The method of claim 13 wherein the lipophilic chemical group is selected from the group consisting of an alkyl, aryl, alkane, ar-alkyl, fatty acid, heterocyclic group, steroid ester, steroid amide, and mixtures thereof.

15. A method of treating a cell for viral infection, and of preventing viral infection in the cell,

the method comprising the step of contacting the cell with the oligonucleotide prodrug of claim 1, the oligonucleotide prodrug comprising a nucleotide sequence complementary to a portion of the nucleic acid of a virus,

the oligonucleotide prodrug entering the cell wherein the esterase or phosphoramidase cleaves the lipophilic chemical group from the nucleotide, thereby releasing the oligonucleotide which binds the complementary portion of the viral nucleic acid,

whereby treating or preventing viral infection is treated or prevented in the cell.

16. The method of claim 15 wherein the lipophilic chemical group is selected from the group consisting of an alkyl, aryl, alkane, ar-alkyl, fatty acid, heterocyclic group, steroid ester, steroid amide, and mixtures thereof.

17. The oligonucleotide prodrug of claim 2, wherein the lipophilic chemical group is an ar-alkyl.

18. The method of claim 13 wherein the lipophilic chemical group is an ar-alkyl.

19. A method of increasing the intracellular or *in vivo* lipid solubility and bioavailability of an oligonucleotide,

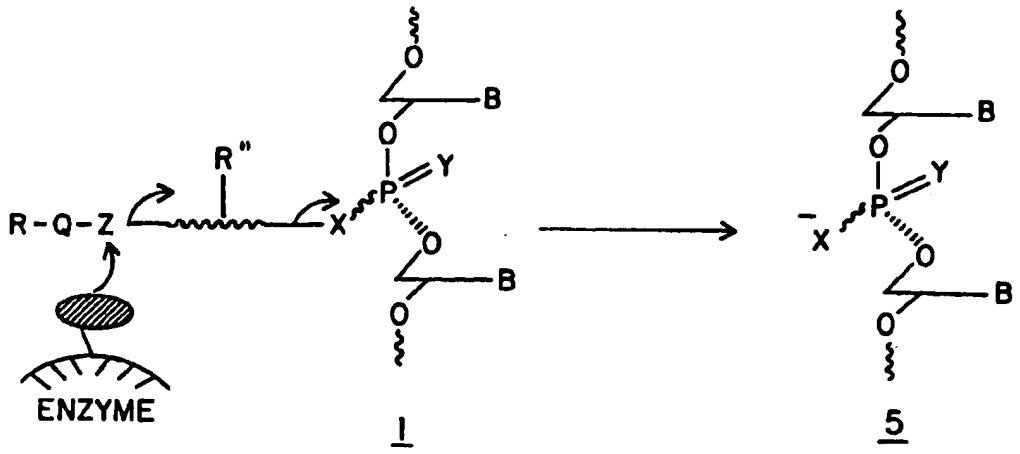
the method comprising the step of derivatizing the oligonucleotide to form an oligonucleotide prodrug which is more lipid soluble and bioavailable than the oligonucleotide,

the prodrug comprising at least six covalently linked nucleotides, at least one nucleotide having a 5'' phosphate, 3' phosphate, or internucleotidic phosphate linkage to which is reversibly and covalently attached a lipophilic chemical group,

the prodrug being reactive with a cellular or tissue enzyme which cleaves the lipophilic group from the derivatized nucleotide, thereby releasing the oligonucleotide in the cell, the enzyme being selected from the group consisting of an esterase and a phosphoramidase.

20. The method of claim 19 wherein the lipophilic chemical group is selected from the group consisting of an alkyl, aryl, alkane, ar-alkyl, fatty acid, heterocyclic group, steroid ester, steroid amide, and mixtures thereof.

21. The method of claim 20 wherein the lipophilic chemical group is an ar-alkyl.



Z = FUNCTIONAL GROUP

R = ALKYL, ARYL, AR-ALKYL, HETEROCYCLIC

R" = BRANCHING GROUP

X = O, S. NR (R = ALKYL, ARYL)

Y = O, S.

Q = HETEROATOM OR COVALENT BAND

FIG. 1

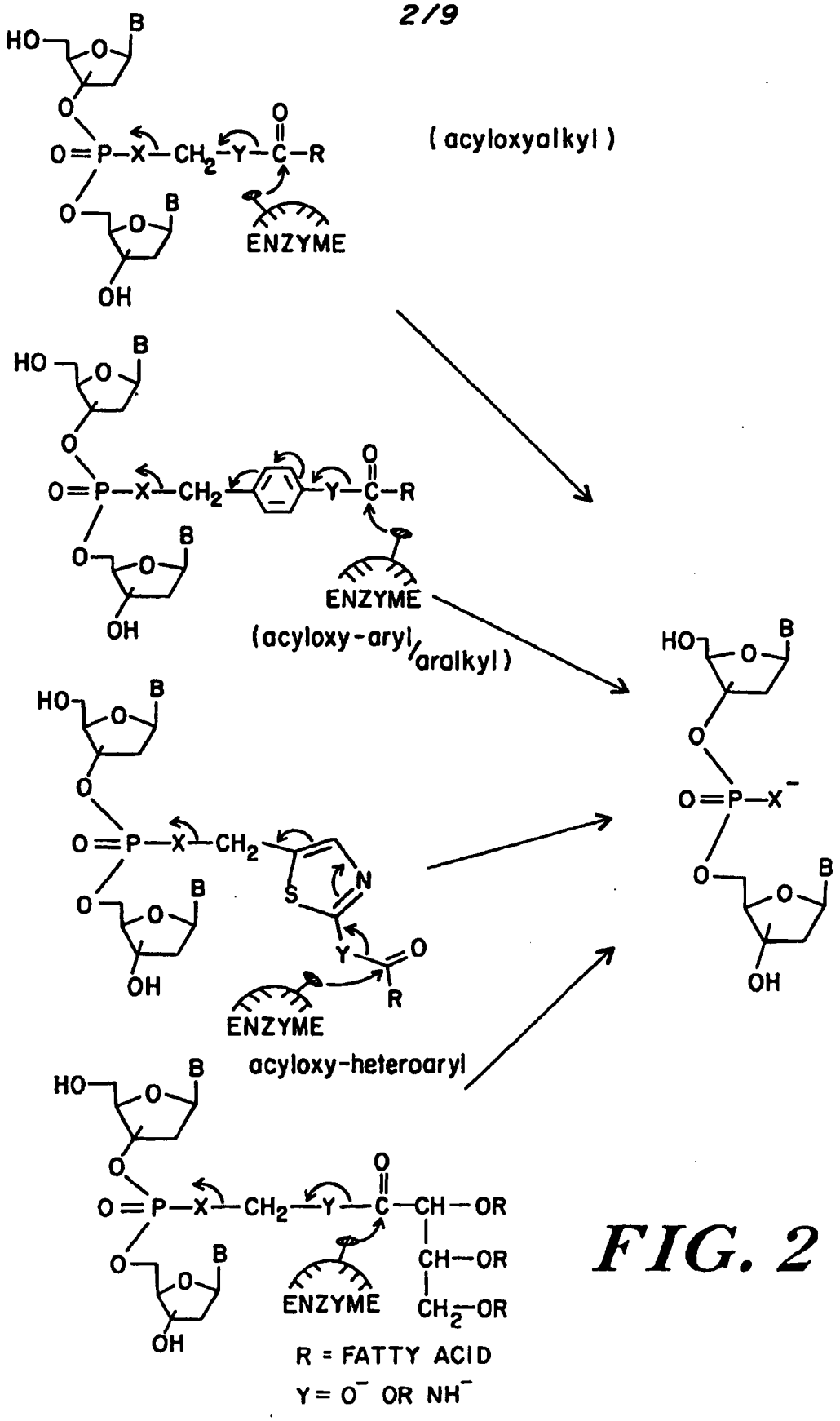
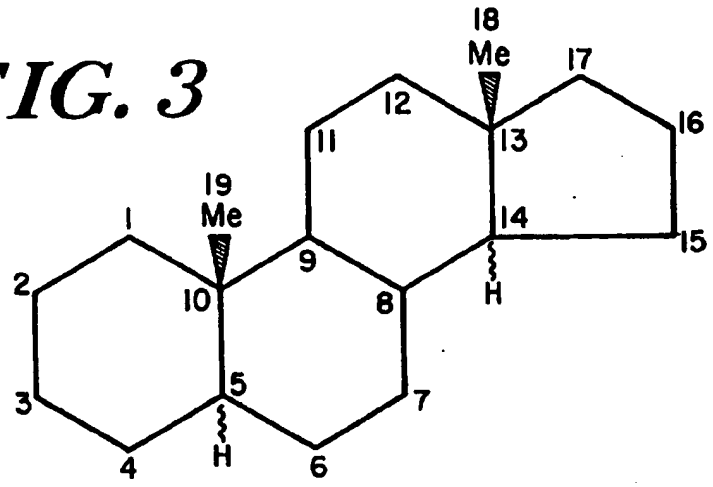
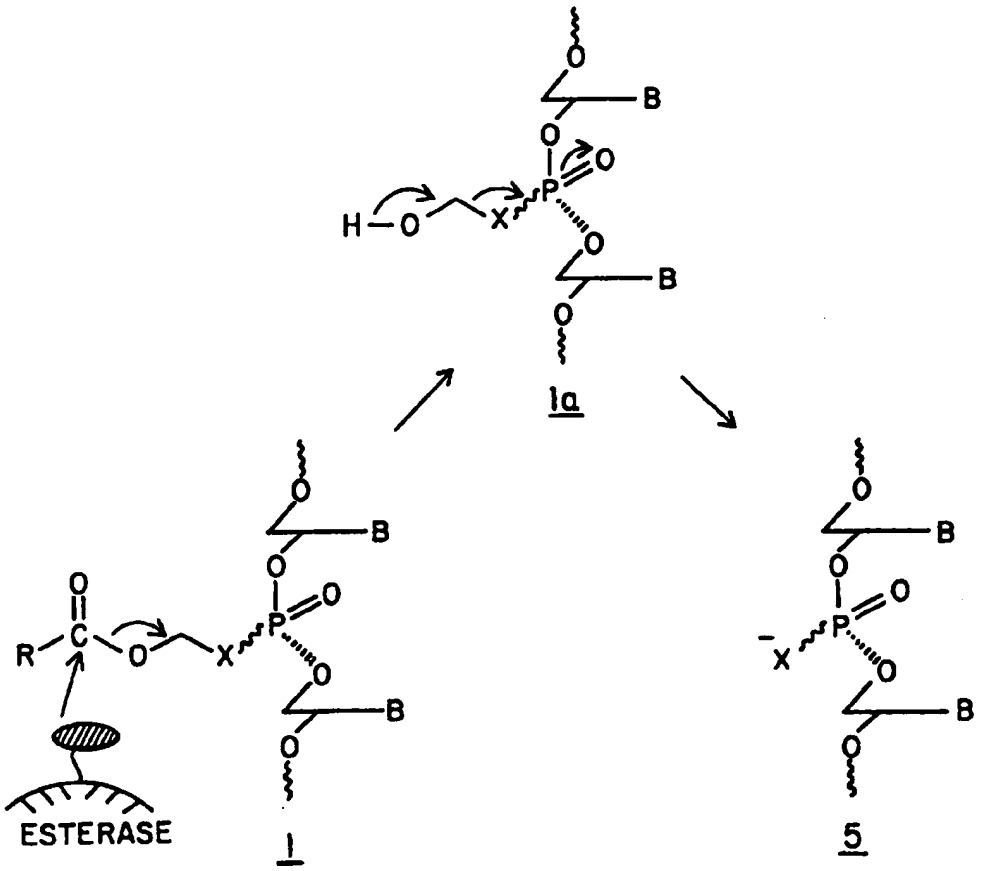

(acyloxyalkyl)
ENZYME
(acyloxy-aryl/aralkyl)
ENZYME
acyloxy-heteroaryl
ENZYME
R = FATTY ACID
Y = O⁻ OR NH⁻

FIG. 2

FIG. 3





X = O, S

FIG. 4

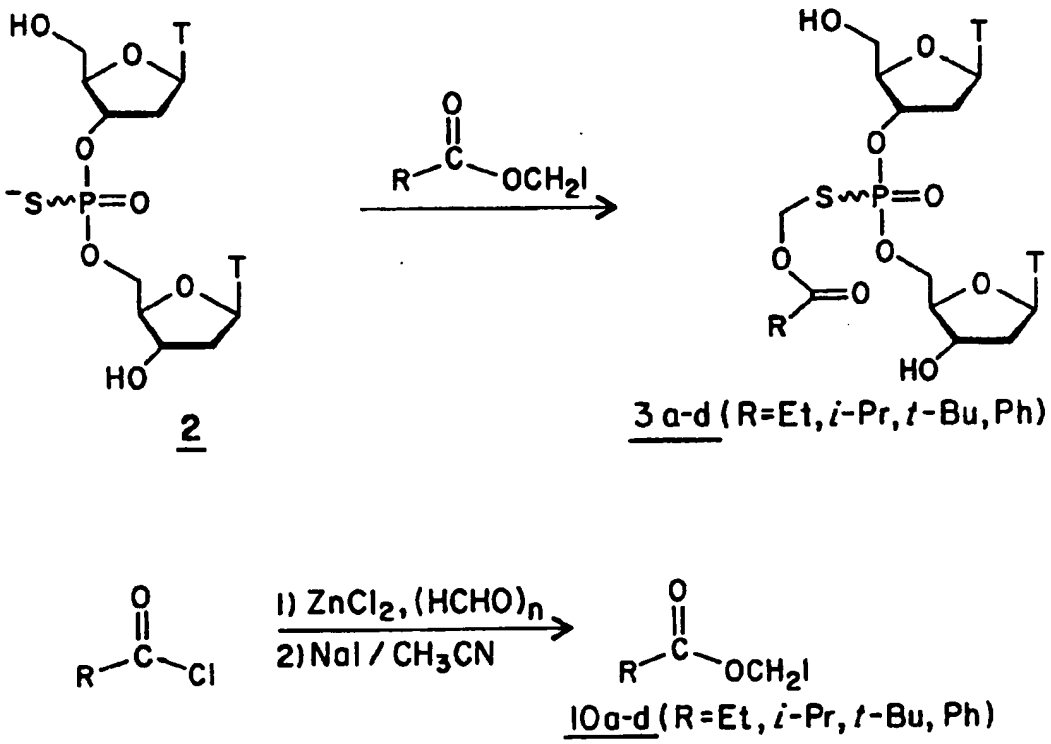

HO
T
O
O
S
P=O
O
O
T
HO
2
R
C
OCH2I
3 a-d (R=Et, i-Pr, t-Bu, Ph)
1) ZnCl2, (HCHO)n
2) NaI / CH3CN
Cl
10a-d (R=Et, i-Pr, t-Bu, Ph)

FIG. 5

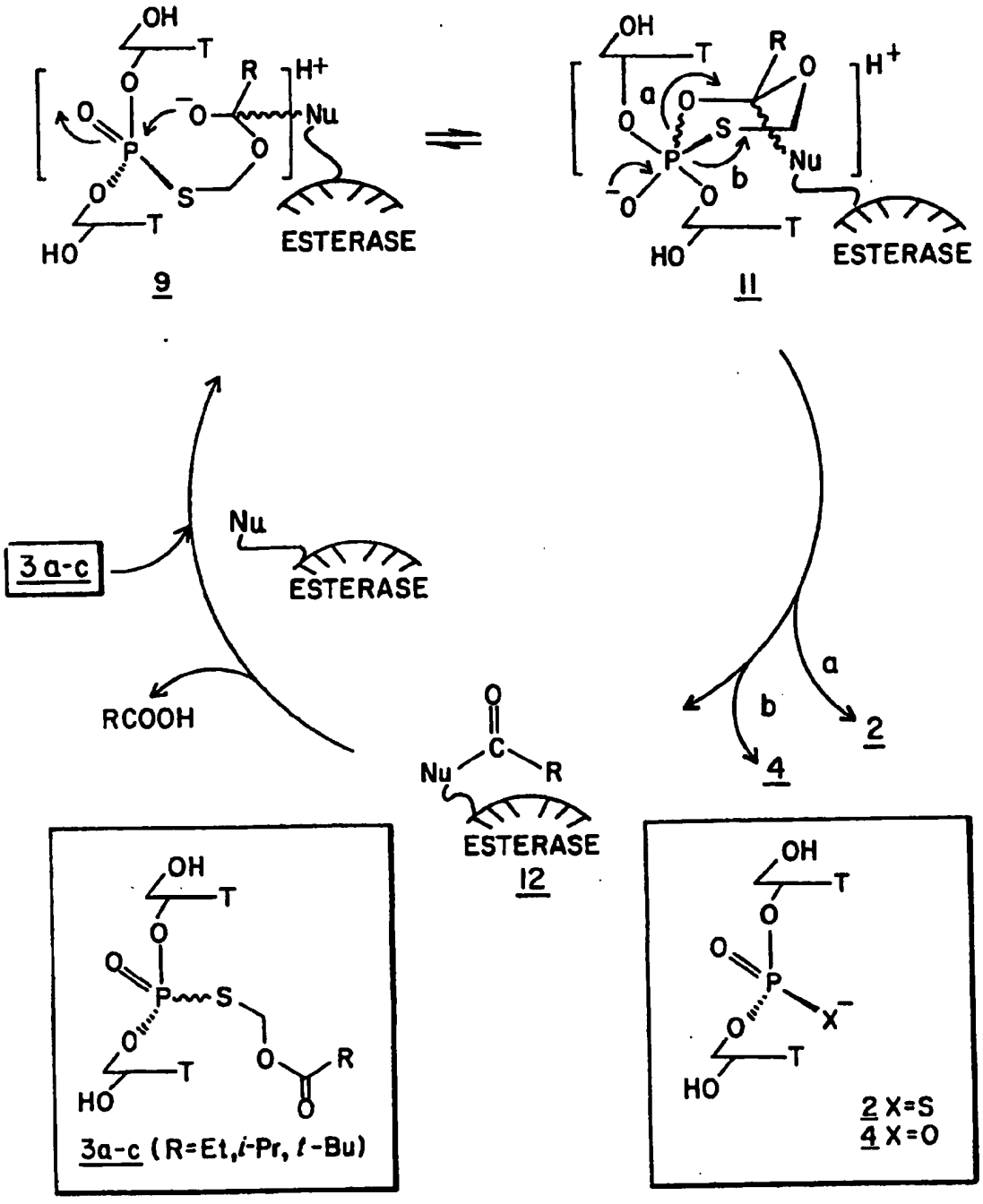

OH
T
O
R
H+
Nu
ESTERASE
HO
9
11
a
b
3a-c
RCOOH
2
4
12
X=S
X=O
3a-c (R=Et, i-Pr, t-Bu)

FIG. 6

HPLC
PROFILES OF HYDROLYSIS OF *Rp* 3b AND *Sp* BY HUMAN SERUM

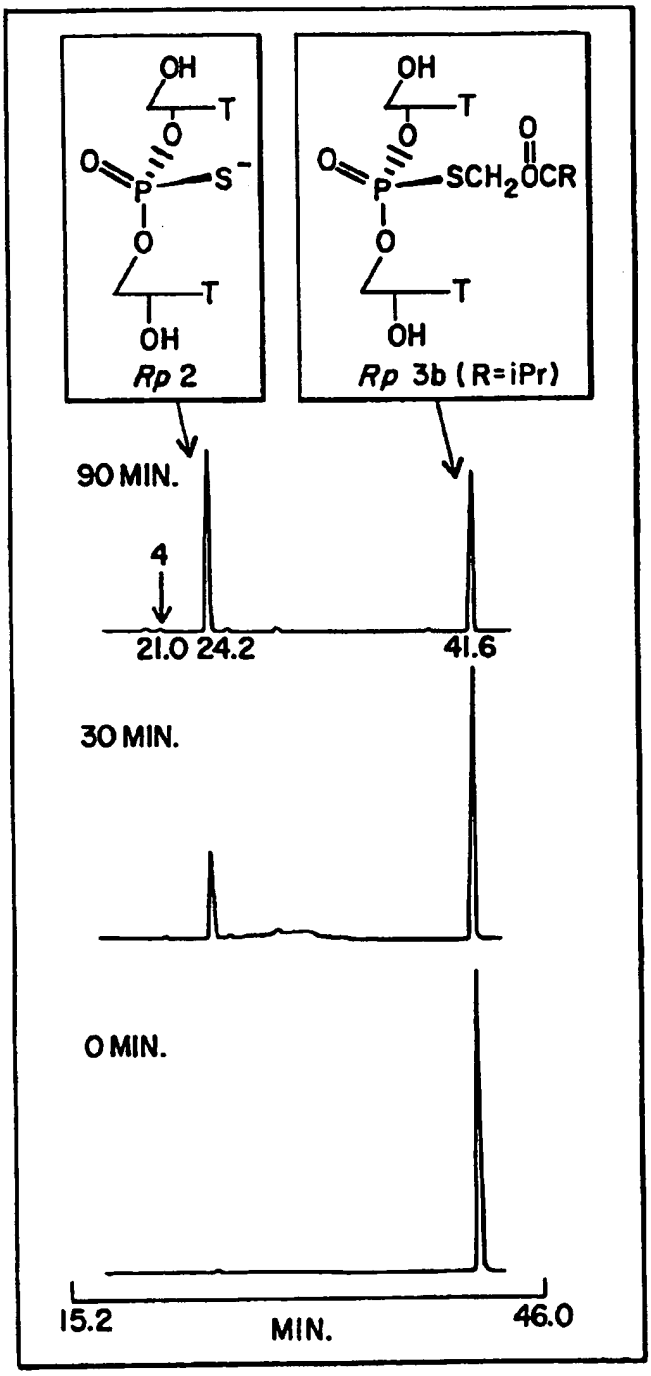


FIG. 7A

HPLC
PROFILES OF HYDROLYSIS OF *Rp* 3b AND *Sp* BY HUMAN SERUM

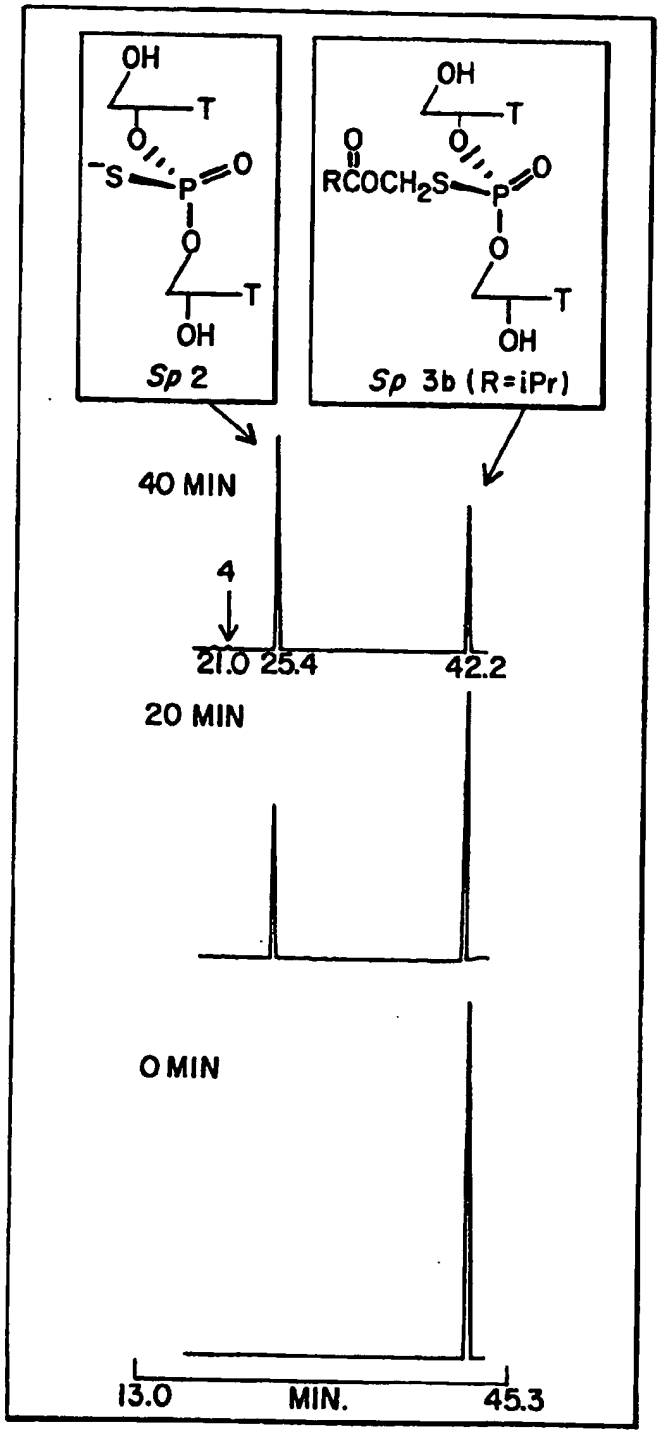


FIG. 7B

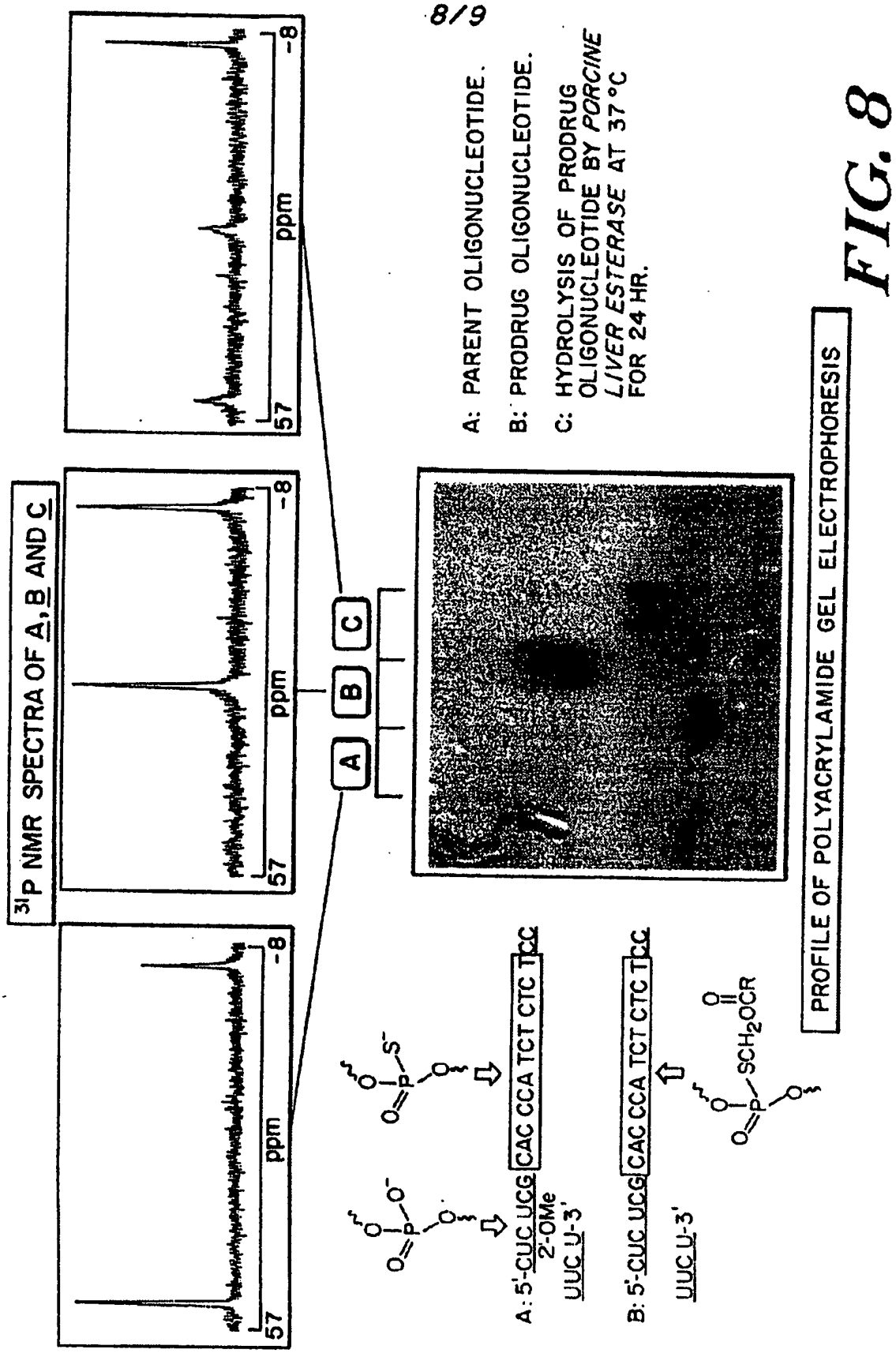

^{31}P NMR SPECTRA OF A, B AND C
ppm
57
-8
A
B
C
A: PARENT OLIGONUCLEOTIDE.
B: PRODRUG OLIGONUCLEOTIDE.
C: HYDROLYSIS OF PRODRUG OLIGONUCLEOTIDE BY PORCINE LIVER ESTERASE AT 37 °C FOR 24 HR.
A: 5'-CUC UCG CAC CCA TCT CTC TCC 2'-OMe UUC U-3'
B: 5'-CUC UCG CAC CCA TCT CTC TCC UUC U-3'
PROFILE OF POLYACRYLAMIDE GEL ELECTROPHORESIS

FIG. 8

FIG. 9